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Kala, Kaia

2009

Kala , K , Haugas , M , Lilleväli , K , Guimera , J , Wurst , W , Salminen , M & Partanen , J 2009 , ' Gata2 is a tissue-specific post-mitotic selector gene for midbrain GABAergic neurons ' , Development , vol. 136 , no. 2 , pp. 253-262 . https://doi.org/10.1242/dev.029900

http://hdl.handle.net/10138/301216
https://doi.org/10.1242/dev.029900

Development 136, 253-262 (2009) doi:10.1242/dev.029900

Gata2 is a tissue-specific post-mitotic selector gene for midbrain GABAergic neurons

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Midbrain GABAergic neurons control several aspects of behavior, but regulation of their development and diversity is poorly understood. Here, we further refine the midbrain regions active in GABAergic neurogenesis and show their correlation with the expression of the transcription factor *Gata2*. Using tissue-specific inactivation and ectopic expression, we show that *Gata2* regulates GABAergic neuron development in the mouse midbrain, but not in rhombomere 1, where it is needed in the serotonergic lineage. Without *Gata2*, all the precursors in the embryonic midbrain fail to activate GABAergic neuron-specific gene expression and instead switch to a glutamatergic phenotype. Surprisingly, this fate switch is also observed throughout the neonatal midbrain, except for the GABAergic neurons located in the ventral dopaminergic nuclei, suggesting a distinct developmental pathway for these neurons. These studies identify *Gata2* as an essential post-mitotic selector gene of the GABAergic neurotransmitter identity and demonstrate developmental heterogeneity of GABAergic neurons in the midbrain.

KEY WORDS: GABA, Interneuron, Midbrain, Neurotransmitter, Mouse, Neurogenesis, Ventral tegmental area (VTA), Serotonin, Dorsal raphe, Rhombomere 1

INTRODUCTION

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain. Neurons producing GABA are located in almost all parts of the central nervous system (CNS) and are highly variable in their morphology, gene expression and projection patterns.

GABAergic neurons are found in several regions of the midbrain. They are thought to operate both as local inhibitory interneurons and as projection neurons with targets elsewhere in the brain. GABAergic neurons are abundant in the dorsal superior colliculi and periaquaductal gray matter, where they are involved in multiple processes, including saccadic eye movements, nociception and defensive behavior (Behbehani et al., 1990; Kaneda et al., 2008). In the ventral midbrain, GABAergic neurons are thought to regulate the activity of the dopaminergic (DA) neurons in the substantia nigra pars compacta and ventral tegmental area (VTA) (Laviolette and van der Kooy, 2004; Tepper and Lee, 2007). In addition to controlling the DA neurons, GABAergic neurons located in the VTA and substantia nigra pars reticulata (SNpr) send their axons to other nuclei in the midbrain, prefrontal cortex and other limbic areas (Laviolette et al., 2004; Fields et al., 2007). Thus, midbrain GABAergic neurons are crucial for neural processes such as the regulation of voluntary and involuntary movements, mood, motivation and addiction.

Despite the functional importance of the midbrain GABAergic neurons, their development remains poorly understood. There appear to be both similarities and differences in the mechanisms that control the development of GABAergic neurons in distinct brain regions. Proliferative progenitor cells of the GABAergic neurons are located in the ventricular zone of both ventral and dorsal midbrain (Tsunekawa et al., 2005). This is in contrast to the forebrain, where the GABAergic neurons are generated in ventral neuroepithelium and reach the cortex by dorsal tangential migration. As in ventral forebrain and other regions of GABAergic neurogenesis, the proneural bHLH transcription factor *Ascl1* (*Mash1*) is expressed in the ventricular zone of the midbrain and plays an important role in GABAergic neuron development (Horton et al., 1999; Casarosa et al., 1999; Miyoshi et al., 2004; Mizuguchi et al., 2006). *Helt* (*Heslike*, *Megane*, *Mgn*) is a bHLH-Orange family transcription factor that is coexpressed with *Ascl1* in the midbrain ventricular zone (Miyoshi et al., 2004). Mice lacking *Helt* function show impaired development of midbrain GABAergic neurons, especially in the dorsal region (Guimera et al., 2006; Nakatani et al., 2007). Furthermore, *Helt* has been shown to regulate the GABAergic versus glutamatergic neuron identity by repressing the proneural genes *Ngn1* and *Ngn2* (*Neurog1* and *Neurog2*), which in turn promote glutamatergic neuron development (Nakatani et al., 2007).

In addition to genes controlling the proliferation, identity and neurogenesis of the ventricular zone progenitor cells, studies of other parts of the CNS have revealed transcription factors that are activated only in the post-mitotic neural precursors. Some of these factors do not regulate the neuronal differentiation process itself, but are needed to select a particular neuronal phenotype from among distinct alternatives and are therefore called selector genes. The bHLH transcription factor *Ptf1a* appears to act as a selector of the GABAergic, as opposed to glutamatergic, fate in the spinal cord and cerebellum (Glasgow et al., 2005; Hoshino et al., 2005; Cheng et al., 2005). However, no such selector gene for the midbrain GABAergic neurons has been identified so far.

Gata2 and Gata3 are related C4 zinc-finger transcription factors. They are involved in the development of several organs and tissues, and have been extensively studied, especially in the hematopoietic system (Tsai et al., 1994). In the developing CNS, *Gata2* and *Gata3* are expressed in similar patterns in distinct brain regions and *Gata2* is often required for the expression of *Gata3* (Nardelli et al., 1999). *Gata2* has been suggested to play a role in the correct development

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Accepted 10 November 2008

of cranial motoneurons (Nardelli et al., 1999), rostral serotonergic neurons in rhombomere 1 (Craven et al., 2004) and spinal V2 interneurons (Zhou et al., 2000; Karunaratne et al., 2002). However, studies of *Gata2* function in neuronal development have been hampered by the early death of *Gata2*-null embryos.

We show here that *Gata2* is specifically expressed in the developing midbrain GABAergic neurons as they exit the cell cycle and differentiate. Using conditional mutagenesis and ectopic expression experiments, we show that *Gata2* acts as an essential post-mitotic selector gene of the GABAergic identity without affecting proliferating neural progenitors or early neurogenic processes in the embryonic midbrain. We further map the GABAergic progenitor domains in the mouse embryonic midbrain and demonstrate region-specific interactions between *Helt* and *Gata2*. Interestingly, our results suggest that GABAergic neurons associated with the ventral DA nuclei in the midbrain use distinct regulatory mechanisms for their development.

MATERIALS AND METHODS

Mice

En1^{Cre} (Kimmel et al., 2000), *Helt*^{KO} [*Mgn*^{tZ} (Guimera et al., 2006)] and *Ascl1*^{KO} [*Mash1*^{null} (Guillemot et al., 1993)] alleles have been described previously. The conditional *Gata2* allele (*Gata2*^{flox}) will be described elsewhere (M.H., K.L. and M.S., unpublished). Briefly, in *Gata2*^{flox}, exons 1-3, encoding the N-terminal half of Gata2, are flanked by loxP sites. Their recombination by Cre recombinase is expected to produce a null allele of *Gata2*. *Gata2*^{cko} embryos were generated from *En1*$^{Cre/+}$; *Gata2*$^{flox/+}$ × *Gata2*$^{flox/flox}$ mouse crosses. For staging, the day of vaginal plug was counted as embryonic day 0.5 (E0.5). For BrdU-incorporation analysis, pregnant females were given an intraperitoneal injection of BrdU (3 mg/100 g body weight) 1-2 hours before dissecting the embryos. All experiments were approved by the Committee of Experimental Animal Research of the University of Helsinki, Finland.

In ovo electroporation

In ovo electroporation was performed at Hamburger-Hamilton stage (HH) 14-16. For Gata2 overexpression, pAdRSVGata2HA plasmid (El Wakil et al., 2006) was microinjected into the embryonic midbrain (third ventricle) together with the EGFP expression vector pEGFP-N3 (Clontech). As a control, embryos were electroporated with the EGFP expression vector only. Electroporation was performed at 20 mV; 10 × 20 millisecond pulses were applied with 500 millisecond intervals. The embryos were harvested 24 or 48 hours later (at HH20-22) and EGFP-expressing embryos were embedded in paraffin for analysis. Five embryos electroporated with pAdRSVGata2HA and two control embryos were analyzed.

In situ mRNA hybridization and immunohistochemistry

Whole-mount mRNA in situ hybridization (ISH) analysis of E10.5 embryos was performed by a modified protocol (Henrique et al., 1995) using a digoxigenin-labeled antisense *Gata2* cRNA probe. For ISH and immunohistochemistry (IHC) on sections, embryos or embryonic brains were fixed in 4% paraformaldehyde (PFA) at room temperature for 1-5 days. Samples were dehydrated and mounted into paraffin. Sections were cut at 5 μm, and adjacent sections were collected on separate slides for parallel stainings. mRNA ISH analyses on paraffin sections were performed as described (Wilkinson and Green, 1990) using ^{35}S- or digoxigenin-labeled cRNA probes. Mouse cDNA probes used for ISH analysis were: *Gata2*, *Gata3* (Lillevali et al., 2004), *Ascl1*, *Fev* (*Pet1*), *Ngn2* (Jukkola et al., 2006), *Gad1* (*Gad67*), *Helt*, *Slc17a6* (*Vglut2*), *Pitx2* (Guimera et al., 2006), *Pou4f1* (gift from Siew-Lan Ang, National Institute of Medical Research, London, UK), *Nkx2-2* (IMAGE clone 480100), *Pax6* (gift from P. Gruss, Max-Planck-Institute for Biophysical Chemistry, Goettingen, Germany), *Isl1* (gift from V. Pachnis, National Institute of Medical Research, London, UK) and *Lmx1b* (gift from H. Simon, Interdisciplinary Centre for Neuroscience, Universität, Heidelberg, Germany). In addition, probes for chicken *Gata3* (Lillevali et al., 2007), *Ngn2* (Matter-Sadzinski et al., 2001), *Slc17a6* and *Gad1* (Cheng et al., 2004) were used.

IHC was performed as described (Kala et al., 2008). The following antibodies were used: guinea pig anti-Heslike (Helt, 1:500; gift from R. Kageyama, Institute for Virus Research, Kyoto University, Japan), goat anti-HA probe (Santa Cruz sc-805-G, 1:500) and anti-Olig2 (Neuromics GT15132, 1:200), mouse anti-BrdU (GE Healthcare RPN20AB, 1:400), anti-HuC/D (Molecular Probes A21271, 1:500), anti-Lim1/2 [Lhx1, Developmental Studies Hybridoma Bank (DSHB) 4F2, 1:10], anti-Mash1 (Ascl1, BD Biosciences 556604, 1:200), anti-Nkx2-2 (DSHB 74.5A5, 1:250), anti-Nkx6-1 (DSHB F55A10, 1:500) and anti-Pax6 (DSHB PAX6, 1:100), rabbit anti-caspase 3 active (R&D Systems AF835, 1:500), anti-Gata2 (Santa Cruz sc-9008, 1:250), anti-5-HT (Immunostar 20080, 1:5000), anti-p57 (NeoMarkers RB-1637-P0, 1:500), anti-phospho-histone H3 (Upstate 06-570, 1:500), anti-Sox2 (Millipore AB5603, 1:500) and anti-TH (Millipore AB152, 1:500).

For combined ISH and IHC, additional primary antibodies were added together with the anti-DIG-POD Fab fragments (Roche). The TSA Fluorescence Palette System (PerkinElmer) was used to visualize ISH signal. Detailed ISH and IHC protocols are available upon request.

Microscopy and quantification

Whole-mount stainings were visualized with a Leica MZFLIII microscope and photographed using an Olympus DP50-CU camera. Stainings on paraffin sections were visualized with an Olympus AX70 microscope and photographed using an Olympus DP70 camera. Images were processed and assembled using Adobe Photoshop software. Red pseudo-color images representing the ISH results were produced by replacing the white signal in dark-field images with red, and overlaying the resulting image with the respective bright-field image.

Confocal images were acquired using the Leica TCS SP5 confocal system and LAS-AF software. Confocal stacks and images were processed and deconvoluted using Imaris 6.1 (Bitplane) and AutoQuantX (AutoQuant) software.

For quantification, cells were counted only from the *Helt* expression domain. For BrdU incorporation, cells from whole neuroepithelium were counted. For phospho-histone H3 expression, only cells lining the ventricle were counted. A standard Student's *t*-test was used for comparing the mean values of the data sets.

RESULTS

Gata2 and Gata3 are expressed in the regions of GABAergic neurogenesis in the embryonic mouse midbrain

Gata2 and *Gata3* are expressed in the developing mouse midbrain, but they have not been associated with any particular neuronal lineage (Nardelli et al., 1999; Zhou et al., 2000). We compared the pattern of expression of *Gata2* and *Gata3* with that of the neural subtype markers *Lmx1b* (DA neurons), *Isl1* (*Islet1*, motoneurons), *Pou4f1* (glutamatergic neurons) and glutamic acid decarboxylase 1 (*Gad1*, GABAergic neurons) in the mouse midbrain from E10.5 to E12.5 by in situ hybridization (ISH) (Fig. 1; see Fig. S1 in the supplementary material). Throughout development, expression of both *Gata2* and *Gata3* coincided with that of *Gad1* and was flanked by *Pou4f1* expression domains (Fig. 1A-L). Strong *Gata2* and *Gata3* ISH signal was detected in the intermediate and marginal zones. Weaker expression of *Gata2*, but no expression of *Gata3*, was also observed in the ventricular zone. We detected no *Gata2* transcripts in *Lmx1b*- or *Isl1*-expressing regions in the mouse midbrain (Fig. 1A-C,M-R). From E12.5 onwards, scattered expression of *Gata2*, *Gata3* and *Gad1* appeared in the dorsal midbrain (Fig. 1C,F,I) correlating with the timing of GABAergic neurogenesis in this region (Tsunekawa et al., 2005).

In order to understand in detail the identity of cells expressing *Gata2*, we compared the expression of Gata2 protein with homeodomain transcription factors expressed in progenitor and precursor cells of the developing midbrain. Lhx1 (Lim1) is

expressed in post-mitotic precursor cells of all midbrain GABAergic neurons in domains m1-m5, as well as in glutamatergic neurons of the red nucleus in m6 [for a description of the midbrain domains m1-m7, see Fig. 8 and Nakatani et al. (Nakatani et al., 2007)]. At E12.5, immunohistochemistry (IHC) demonstrated coexpression of Gata2 and Lhx1 in the intermediate zone cells of domains m3-m5, where GABAergic neurons are produced at this stage of development (Fig. 1S,T). Nkx6-1 and Nkx2-2 are expressed in specific subsets of GABAergic neuron progenitors and precursors (Nakatani et al., 2007). Colocalization of Gata2 and Nkx6-1 was observed in the basal ventricular zone of domains m5 and m3 (Fig. 1U; see Fig. S1A in the supplementary material). Also, some progenitor/precursor cells in m4 expressed both Nkx2-2 and Gata2 (Fig. 1V; see Fig. S1B in the supplementary material). Thus, we found Gata2 expression in all the midbrain regions that give rise to GABAergic neurons.

We observed a small area that was negative for *Gata2*, *Gata3* and *Gad1* expression in the m4 domain expressing *Nkx2-2* (Fig. 1B,C,E,F,H,I). *Pax6* is also expressed near the *Nkx2-2*-positive region in the developing midbrain (Ahsan et al., 2007). By combining ISH and IHC, we showed that *Pax6* is expressed in a subset of Nkx2-2-positive cells in the ventral m4 domain at E11.5 (see Fig. S1C in the supplementary material). Interestingly, no Gata2 protein was detected in the Pax6-expressing cells (Fig. 1W). Next, we analyzed the neurotransmitter identities in the m4 marginal zone. Both ISH and IHC demonstrated that the Pax6-positive cells express *Slc17a6* [vesicular glutamate transporter 2 (*Vglut2*)] and are negative for *Gad1* (see Fig. S1D-G in the supplementary material). Thus, the m4 domain can be further divided into a GABAergic Gata2-positive dorsal part (m4-D) and a glutamatergic Pax6-positive ventral part (m4-V). In addition to the m4-V, putative glial progenitors expressing Olig2 were also devoid of Gata2 expression at E12.5 (Fig. 1X). In conclusion, the expression of *Gata2* mRNA and protein correlates with the spatial and temporal appearance of the GABAergic neuron lineage in the developing mouse midbrain.

Gata2 is activated in the developing GABAergic neurons as the progenitor cells exit the cell cycle and start to differentiate

To gain insight to the possible role of *Gata2* in GABAergic neurogenesis, we compared the expression of the bHLH transcription factors Ascl1 and Helt with that of Gata2 in the m3-m5 ventricular zone by IHC (Fig. 2A-C). As reported, extensive coexpression of Ascl1 and Helt was observed in the progenitors (Fig. 2D) (Miyoshi et al., 2004). Notably, we observed coexpression mostly at the apical and medial regions of the ventricular zone, while on the basal side, the Ascl1-expressing nuclei were negative for Helt. Also, Gata2 was detected in the Ascl1-positive cells (Fig. 2F), but, in contrast to Helt, its expression was detected primarily in the nuclei on the basal side of the ventricular zone (Fig. 2E,F). In the medial ventricular zone, nuclei coexpressing Helt and Gata2 were detected (Fig. 2E). The gradual upregulation of Gata2 in the Ascl1- and Helt-expressing cells/nuclei as they move further away from the ventricular surface suggested that Gata2 expression might be switched on in the GABAergic progenitors that are about to exit the ventricular zone. To confirm this, we analyzed the Gata2-expressing cells for expression of HuC/D (Elavl3/4), a marker of post-mitotic neurons, and for DNA

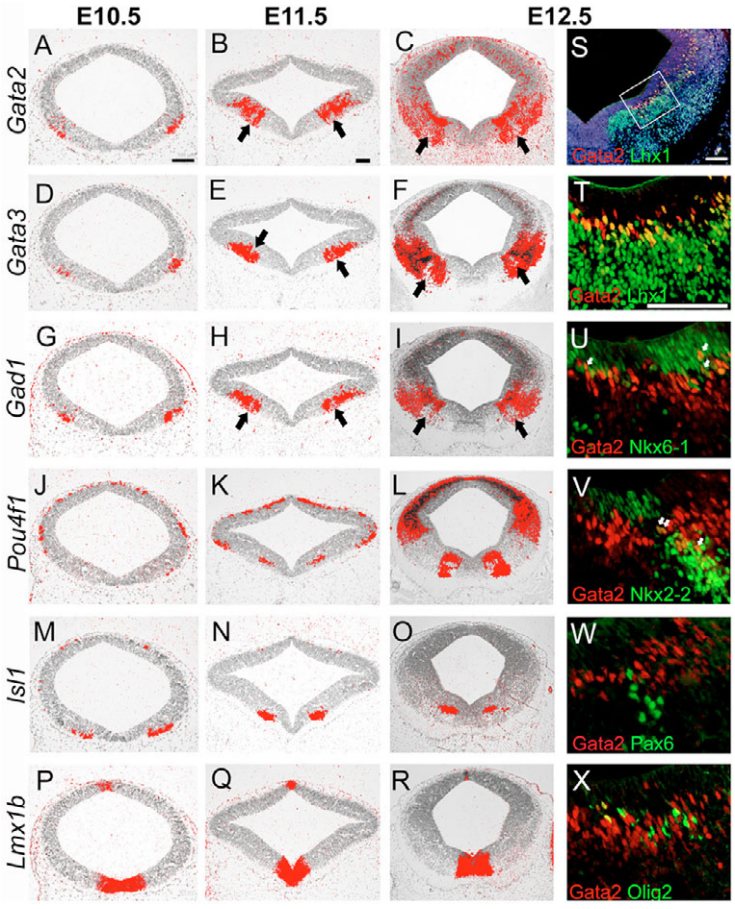

Fig. 1. *Gata2* and *Gata3* expression in embryonic mouse midbrain compared with expression of neural subtype markers. (**A-R**) In situ hybridization (ISH) with *Gata2*, *Gata3*, *Gad1*, *Pou4f1*, *Isl1* and *Lmx1b* probes on coronal sections of wild-type embryos. The embryonic stages are indicated at the top of each column. Black arrows point to the negative areas within the expression domains of *Gata2*, *Gata3* and *Gad1*. (**S-X**) Co-immunostaining for Gata2 (red) and Lhx1, Nkx6-1, Nkx2-2, Pax6 or Olig2 (green). The boxed area in S indicates the region enlarged in T-X. White arrows indicate examples of Gata2 and Nkx6-1 (U), and Gata2 and Nkx2-2 (V), coexpressing cells. Scale bars: 100 μm.

synthesis as measured by BrdU incorporation. Representing mostly intermediate zone cells, a large proportion of Gata2-expressing cells were also positive for HuC/D (Fig. 2G, arrows). In addition, HuC/D-negative, yet Gata2-expressing cells were observed in the ventricular zone (Fig. 2G, arrowheads). However, these Gata2-expressing nuclei did not incorporate BrdU during a short labeling pulse (Fig. 2H). Together, our results show that Gata2 expression is switched on in GABAergic progenitors as they become post-mitotic precursor cells and start to differentiate.

Helt promotes Gata2 expression

Because of the sequential expression of *Helt* and *Gata2* in the GABAergic progenitor and precursor cells, we asked whether *Helt* is required for *Gata2* activation. We analyzed *Helt*-null embryos [$Mgn^{tZ/tZ}$ (Guimera et al., 2006)] for *Gata2* mRNA expression. Interestingly, *Gata2* was undetectable in E11.5 and E13.5 $Helt^{KO}$ midbrain, except for the ventral-most GABAergic neurons (Fig. 3A,B; data not shown). The remaining *Gata2* expression correlated with *Gad1* expression (Fig. 3C,D, arrow). We detected no *Gata2* or *Gad1* expression in the dorsal midbrain of the E13.5 $Helt^{KO}$ embryos (data not shown). At the same time, *Ascl1* expression was unaffected by *Helt* inactivation (Fig. 3E,F), as shown previously (Nakatani et al., 2007).

To test whether *Ascl1* also regulates *Gata2* expression, we analyzed E11.5 *Ascl1*-null mutants (Guillemot et al., 1993). In these embryos, expansion of the Sox2- and Helt-expressing layer suggested a failure in cell cycle exit of GABAergic progenitors (Fig. 3I-L). However, Gata2 was still expressed in regions where post-mitotic precursors were produced (Fig. 3G,H). Altogether, except for the ventral-most GABAergic precursors, activation of *Gata2* requires *Helt*, but does not require *Ascl1*.

Conditional *Gata2* inactivation leads to specific loss of GABAergic neuron precursors in the embryonic midbrain

To study the role of *Gata2* in GABAergic neuron development, we inactivated *Gata2* in the mouse midbrain and rhombomere 1 (r1) by crossing mice carrying a conditional allele of *Gata2* (M.H., K.L. and M.S., unpublished) with those carrying the $En1^{Cre}$ allele (Kimmel et al., 2000). In the $En1^{Cre}$ mouse strain, the Cre recombinase activity has been demonstrated as early as the 5-10 somite stage (Chi et al., 2003; Trokovic et al., 2003), well before the expression of *Gata2* in the midbrain-r1 region. In the $En1^{Cre}$; $Gata2^{flox/flox}$ ($Gata2^{cko}$) mice, no *Gata2* expression was detected in the midbrain-r1 region at E10.5 or E11.5 (see Fig. S2 in the supplementary material).

Interestingly, we observed a complete loss of *Gad1*, *Gad2* and *Gata3* expression in $Gata2^{cko}$ midbrain at E11.5 and E13.5 (Fig. 4A,A′,B,B′,E,F; see Fig. S3A-H in the supplementary material). No GABAergic neuron precursors were detected at any dorsoventral or anteroposterior level in the midbrain of $Gata2^{cko}$ mutants at these stages. These results suggested an early and absolute requirement for *Gata2* in midbrain GABAergic neuron development that cannot be compensated for over time.

Unaltered progenitor cell proliferation, patterning, neurogenesis, survival and cell cycle exit in the $Gata2^{cko}$ mutant midbrain

Next, we investigated which steps in midbrain GABAergic neuron development are affected by the loss of *Gata2*. Despite the loss of GABAergic precursors, there were no overt anatomical defects in the embryonic $Gata2^{cko}$ mutant midbrain. Nevertheless, we analyzed in more detail the properties of the proliferative progenitor

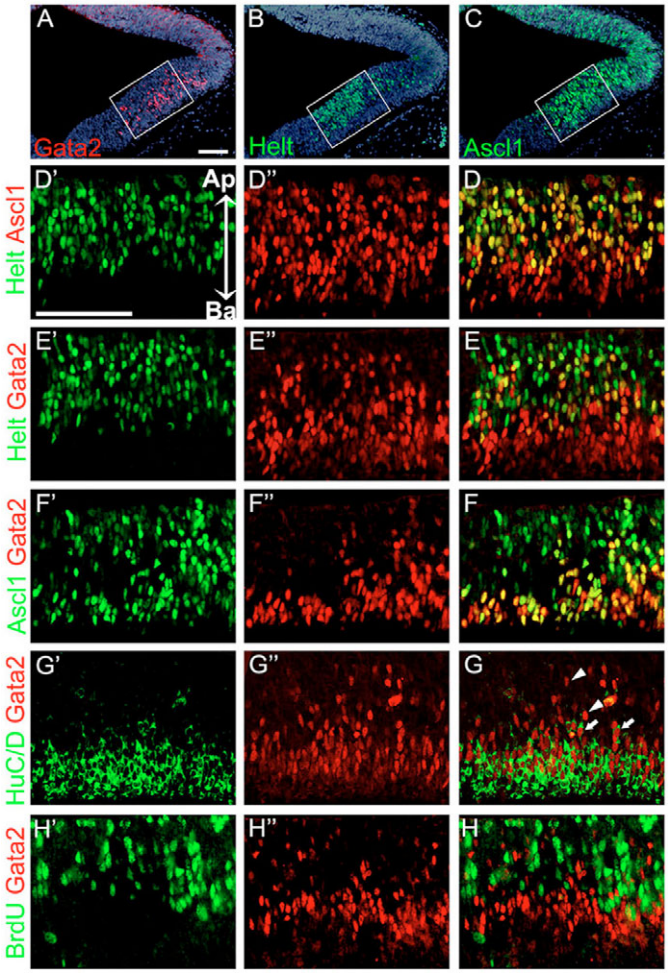

Fig. 2. Expression of Gata2 during GABAergic differentiation. (**A-F″**) Relationship between Gata2, Helt and Ascl1 expression in GABAergic progenitors analyzed by immunohistochemical co-staining. The boxed area in A-C is enlarged in D-H″. Nuclei at the apical (Ap) side of the ventricular zone abundantly coexpress Helt (D′) and Ascl1 (D″). Nuclei near the basal (Ba) side of the ventricular zone coexpress Ascl1 (F′) and Gata2 (F″). Nuclei coexpressing Helt (E′) and Gata2 (E″) are detected mostly in the medial ventricular zone. (**G-H″**) Analysis of post-mitotic differentiation of the Gata2-positive cells. Both HuC/D-positive (arrows) and -negative (arrowheads) cells expressing Gata2 can be detected (G). Ventricular zone cells expressing Gata2 do not incorporate BrdU during a 1.5-hour labeling pulse (H). Scale bars: 100 μm.

cells that give rise to the midbrain GABAergic precursors. In the $Gata2^{cko}$ mutants, the ventricular zone progenitors still expressed Sox2 and there was no difference in the thickness of the Sox2-positive ventricular zone or HuC/D-positive marginal zone compared with the wild type (see Fig. S4A-E in the supplementary material). Consistently, we detected no major changes in the numbers of phospho-histone H3-positive mitotic nuclei or BrdU-incorporating S-phase nuclei in the GABAergic progenitor domain of the $Gata2^{cko}$ mutants (see Fig. S4F-K in the supplementary material). Also, we observed no increase in apoptotic cell numbers (see Fig. S4N,O in the supplementary material). Thus, we conclude that the loss of GABAergic neurons is not due to impaired proliferation or survival of their progenitors.

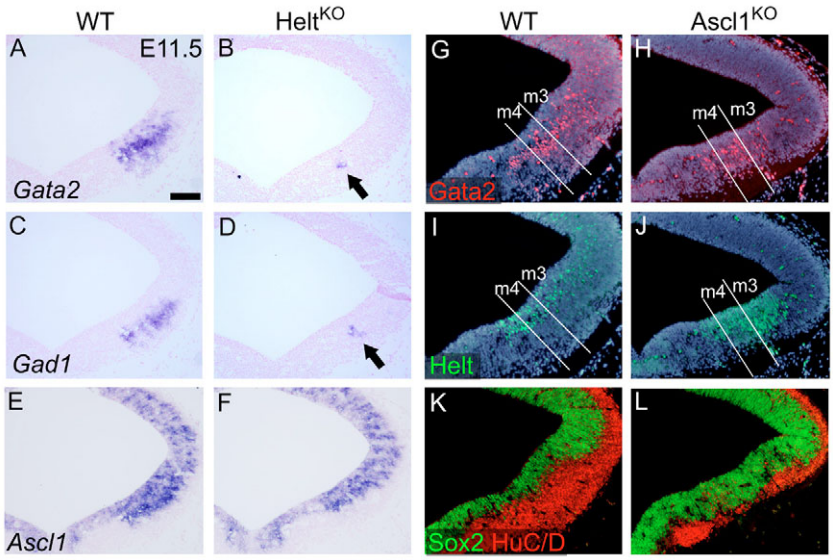

Fig. 3. Gata2 and GABAergic marker gene expression in *Helt*-null and *Ascl1*-null mouse embryos. (**A-F**) ISH analysis of *Gata2*, *Gad1* and *Ascl1* expression on coronal sections of E11.5 WT and *Helt*^{KO} midbrains. Arrows indicate the ventral population of GABAergic neurons that still develop in the *Helt*-null mutants. (**G-J**) Immunohistochemistry (IHC) of Gata2 and Helt expression in E11.5 wild-type (WT) and *Ascl1*-null embryos. (**K**,**L**) Sox2 and HuC/D co-IHC demonstrates delayed neurogenesis in the *Ascl1*-null embryo, especially in the m3 GABAergic domain. The borders of the m3 region were deduced from Nkx2-2 IHC signal on adjacent sections. Scale bar: 100 μm.

We then characterized patterning and neurogenesis in the GABAergic neuron progenitors of the *Gata2*^{cko} mutants. All the analyzed transcription factors, including *Nkx6-1*, *Nkx2-2* and *Pax7*, involved in the patterning of the ventricular zone progenitor cell layer were correctly expressed (Fig. 4K,L,Q-T; data not shown). The transcription factors showing normal expression in the progenitors also included *Ascl1* and *Helt* (Fig. 4M,N,I,J), as well as *Ngn2*, which has been suggested to be involved in glutamatergic neurogenesis in the midbrain (Fig. 4O,P). In addition, we observed no change in the expression of p57 (Cdkn1c), a cell cycle inhibitor upregulated at withdrawal from the cell cycle, demonstrating that *Gata2*-deficient progenitors are still able to exit from the cell cycle and become post-mitotic precursors (see Fig. S4L,M in the supplementary material). Our results suggest that progenitor cell patterning and neurogenic cell cycle exit are not disturbed by the loss of *Gata2* in the midbrain.

GABAergic-to-glutamatergic fate transformation of the post-mitotic precursors in the midbrain of *Gata2*^{cko} mutants

Since the GABAergic progenitors were unaffected in *Gata2*^{cko} midbrain, we analyzed the characteristics of the post-mitotic neuronal precursors. In contrast to the normal appearance of the GABAergic progenitor cell layer, all the post-mitotic derivatives of these cells had transdifferentiated into a glutamatergic phenotype in the *Gata2*^{cko} midbrain. In E11.5 *Gata2*^{cko} mutants, the expression of *Slc17a6* expanded to cover the entire marginal zone, including the regions where the GABAergic precursors should reside (Fig. 4C,C′,D,D′). In addition, we detected ectopic expression of *Pou4f1* in the post-mitotic cells of the m3 domain (Fig. 4G,H), indicating that in the absence of *Gata2*, differentiating neurons in m3 could have acquired a glutamatergic neuron fate typical for the adjacent m2. Ventrally in the m5 domain, Nkx6-1 is downregulated during post-mitotic differentiation of wild-type GABAergic precursor cells. We noticed continued Nkx6-1 expression in the m5 marginal zone in the E11.5 *Gata2*^{cko} mutants, similar to that in the neighboring glutamatergic domain m6 (Fig. 4S,T). However, the fate transformation is incomplete because *Pou4f1*, another marker of m6, was not activated in the mutant m5 domain (data not shown).

In addition to *Gad1*, *Gad2* and *Gata3*, other genes characteristic of the post-mitotic GABAergic neuron precursors were also downregulated in the *Gata2*^{cko} mutants. At E11.5, Lhx1 was downregulated in the m5 and m3 domains, but persisted in the m4 (Fig. 4U,V). In addition, the glutamatergic domain m6 continued to express Lhx1, as expected. Although some Lhx1-positive cells appeared in the marginal zone of m5 (Fig. 4V), they might have originated from neighboring domains, as the intermediate zone of m5 is negative for Lhx1 but both m4 and m6 continued to express it. The m4 domain also appeared to retain its identity as judged by continued *Nkx2-2* expression and lack of *Pou4f1* expression in the *Gata2*^{cko} mutants (Fig. 4K,L,G,H). Despite continued *Lhx1* and *Nkx2-2* expression, there was complete loss of GABAergic markers and uniform glutamatergic marker gene expression in the m4 domain of the mutants. This discrepancy could be explained by an uncoupling of regional patterning and neurotransmitter selection, as suggested by Nakatani et al. (Nakatani et al., 2007). Alternatively, because m4 produces both GABAergic and glutamatergic precursors (see above), there could be a transformation within m4 whereby the GABAergic m4 precursors (m4-D) assume an identity of glutamatergic m4 precursors (m4-V, Fig. 4A,C). To test this, we analyzed Pax6 expression in the *Gata2*^{cko} mutants. Ectopic Pax6-expressing cells were observed in the m4-D region (Fig. 4W,X) indicating that in the absence of *Gata2*, the whole m4 domain acquired a fate similar to m4-V glutamatergic neurons. Thus, *Gata2* is essential for activating gene expression patterns typical for post-mitotic GABAergic precursor cells in the midbrain. Without *Gata2*, the precursor cells appear to adopt a phenotype resembling, but not necessarily identical to, that of the adjacent glutamatergic regions.

Ectopic Gata2 is sufficient to induce GABAergic differentiation in embryonic chicken midbrain

We next examined whether *Gata2* alone is sufficient to switch on the GABAergic differentiation pathway. For this, we employed ectopic expression of *Gata2* in chicken embryos. First, we studied the pattern of endogenous chicken *Gata2* (*cGata2*) expression and its relationship with the development of GABAergic neurons in chick midbrain. As in the mouse embryo at the equivalent stage, expression of *Helt*, Gata2, *cGata3*, Lhx1 and *cGad1* coincided in the ventrolateral chick midbrain, and was flanked by *cNgn2*- and *cSlc17a6*-expressing glutamatergic domains at HH20-22 and HH22-24 (Fig. 5A-I). Similar to in

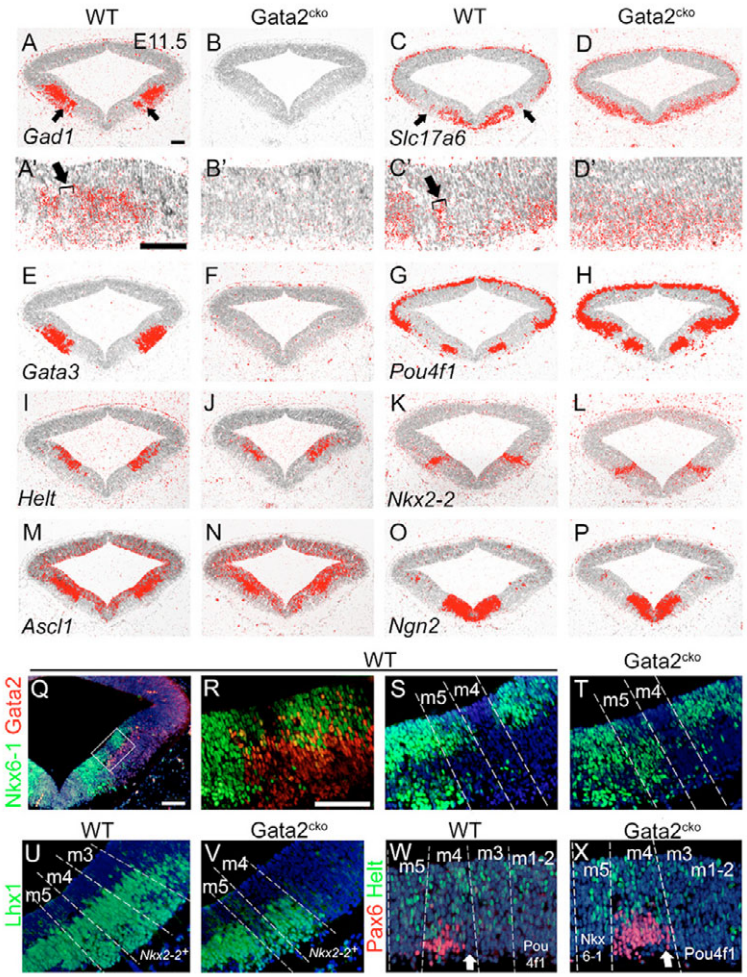

Fig. 4. Fate transformation of midbrain GABAergic neuron precursors in *Gata2^cko^* mouse embryos. (**A-P**) ISH analysis of *Gad1*, *Slc17a6*, *Gata3*, *Pou4f1*, *Helt*, *Nkx2-2*, *Ascl1* and *Ngn2* expression on coronal sections of E11.5 wild-type (WT) and *Gata2^cko^* midbrains. (A′-D′) Higher magnification views from the GABAergic neurogenesis domain. Black arrows point to the small group of *Slc17a6*-expressing glutamatergic neurons in m4 (C,C′) surrounded by *Gad1*-expressing GABAergic neurons (A,A′) in WT embryos. (**Q-X**) IHC analysis of transcription factors Nkx6-1, Lhx1 and Pax6 in the m3-m5 domains of E11.5 WT and *Gata2^cko^* midbrain. The borders of the m4 domain (S-X) were deduced from the borders of *Nkx2-2* ISH or IHC signal on adjacent sections. The ventral border of m5 was deduced from the ventral border of Helt expression. The border between m3 and m1-2 (W) was deduced from the ventral border of Pou4f1 IHC on an adjacent section. White arrows (W,X) point to the dorsal part of m4 (m4-D). Scale bars: 100 μm.

E12.5 mouse midbrain, we observed scattered *cGad1* expression in the chick dorsal midbrain at a slightly later stage (HH22-24, Fig. 5H). Unlike in the mouse, dorsal *cNgn2* expression was detected primarily in the marginal zone and might mark the differentiated glutamatergic neurons rather than their progenitors (Fig. 5C). However, our results demonstrate that overall, the pattern, timing and regulatory mechanisms of midbrain GABAergic neuron production are likely to be conserved between these two species.

We then used tissue electroporation to overexpress *Gata2* in the dorsal chick midbrain, which normally gives rise to both GABAergic and glutamatergic neurons. Abundant Lhx1-, *cGata3*- and *cGad1*-positive cells were observed in the regions expressing ectopic *Gata2*, 24 and 48 hours after electroporation (Fig. 5J-M,O,P; data not shown). No ectopic expression of GABAergic markers was observed on the control side not expressing ectopic *Gata2* or in embryos electroporated with the GFP-expressing control vector only (data not shown). At the same time, expression of *cNgn2* was reduced dorsally (Fig. 5N,Q). Given the pattern of *cNgn2* expression (see above), this might reflect a specific failure in the production of glutamatergic neurons, rather than a defect in their ventricular zone progenitors. However, general developmental retardation of the *Gata2*-electroporated tissue might also contribute to *cNgn2* downregulation. In all, these results suggest that *Gata2* is sufficient to induce a GABAergic phenotype, as judged by c*Gad1*, *cGata3* and Lhx1 expression, in the midbrain neuroepithelium.

Normal development of GABAergic, but loss of serotonergic, neurons in rhombomere 1 of *Gata2^cko^* mutants

To study GABAergic neuron development in r1, we analyzed E11.5 wild-type and *Gata2^cko^* mouse embryos for *Gata3* and *Gad1* expression. In striking contrast to the midbrain, where both genes were completely downregulated, *Gata3* and *Gad1* expression in the GABAergic precursors of r1 was not altered in the mutant (Fig. 6A,B,E,F). Thus, *Gata2* is dispensable for the early development of GABAergic neurons in r1.

Previous studies have suggested a role for *Gata2* and *Gata3* in the development of other neuronal populations in the midbrain-r1 region, including cranial motoneurons and serotonergic neurons (Nardelli et al., 1999; Craven et al., 2004; Pattyn et al., 2004). Consistent with the pattern of *Gata2* expression, we observed no defects in the cranial *Isl1*-expressing motoneurons of nIII and nIV in the *Gata2^cko^* mutants (see Fig. S3I,J in the supplementary material). Thus, the nIII/nIV defects in the *Gata2*-null mutants were likely to be secondary to other developmental abnormalities in these embryos. By contrast, almost no serotonin (5-HT)-, *Lmx1b*- or *Fev* (*Pet1*)-positive serotonergic neurons were detected in r1 of E11.5 and E13.5 *Gata2^cko^* mutants (Fig. 6C,D,G-L). Despite its continued expression in the GABAergic precursors, *Gata3* was specifically downregulated in the serotonergic neuron precursors in r1 (Fig. 6A,B). These results confirm that *Gata2* is required for the development of rostral serotonergic neurons. They also further demonstrate the loss of *Gata2* function in r1 of *Gata2^cko^* mutants.

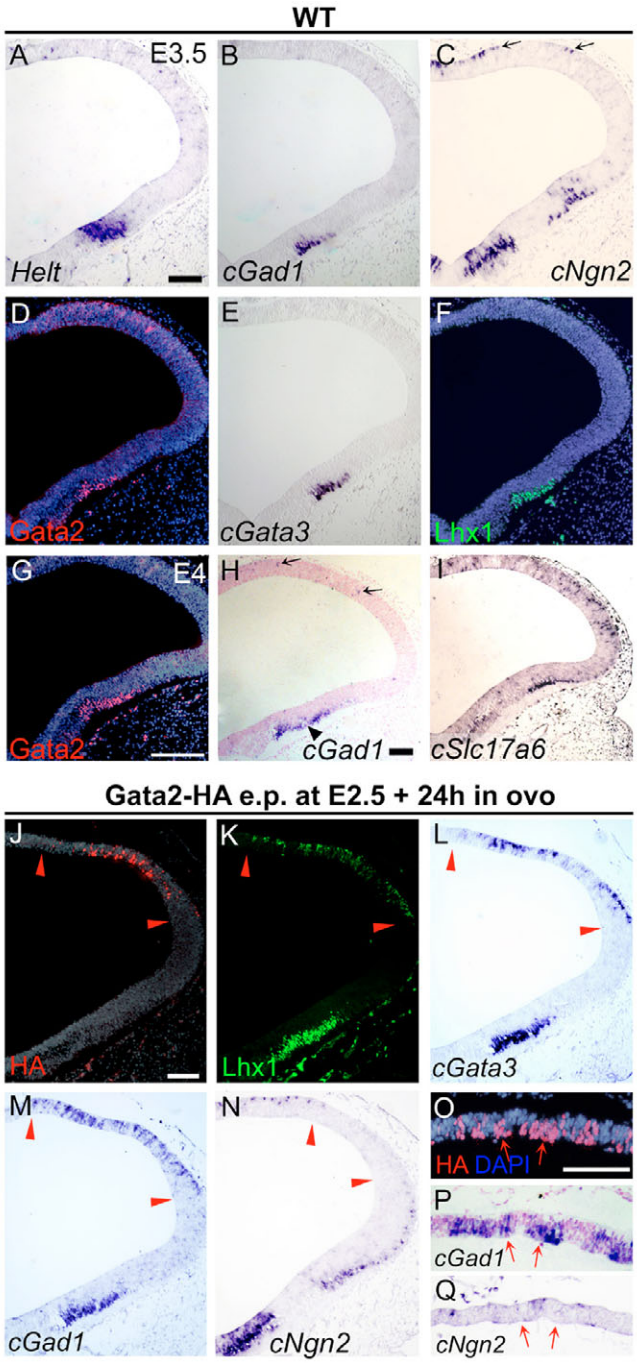

Fig. 5. Induction of GABAergic differentiation in chick embryonic midbrain by ectopic *Gata2*. (**A-I**) The expression patterns of *Helt* (ISH), *cGad1* (ISH), *cNgn2* (ISH), Gata2 (IHC), *cGata3* (ISH), Lhx1 (IHC) and *cSlc17a6* (ISH) in coronal sections of E3.5 (A-F) and E4 (G-I) embryonic chick midbrain (c, chicken). Arrows in C point to *cNgn* expression in the marginal zone of dorsal midbrain. Similar to in mouse midbrain, a gap was observed in ventrolateral *cGad1* expression (arrowhead in H). Scattered *cGad1* expression appears in the dorsal midbrain by E4 (arrows in H). (**J-Q**) Electroporation of Gata2-HA expression vector into chick dorsal midbrain. Expression of the electroporated Gata2 is detected by anti-HA antibody (J,O). Expression of *cGad1* (M,P), *cGata3* (L) and *cNgn2* (N,Q) in the electroporated area was detected by ISH. Induction of Lhx1 was detected by IHC (K). Arrowheads (J-N) mark the electroporated area expressing ectopic Gata2. Red arrows (O-Q) point to the cells that express ectopic Gata2 (HA-positive). Scale bars: 100 μm.

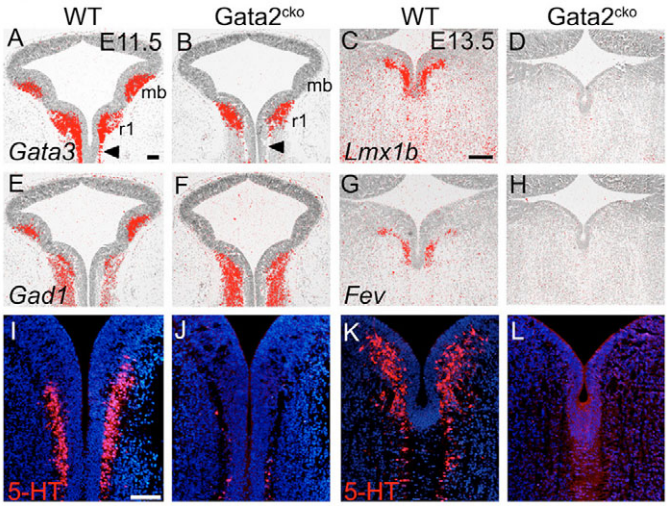

Fig. 6. Development of r1 GABAergic and serotonergic neurons in *Gata2^{cko}* mouse embryos. ISH with *Gata3* (**A**,**B**) and *Gad1* (**E**,**F**) probes on adjacent coronal sections of E11.5 wild-type (WT) and *Gata2^{cko}* embryos. Midbrain (mb) and rhombomere 1 (r1) regions are indicated. The arrowhead points to *Gata3* expression in the serotonergic neuron precursors (A). This *Gata3* expression domain is lost in the *Gata2^{cko}* mutants (arrowhead in B). Anti-serotonin (5-HT) IHC on coronal sections of E11.5 WT (**I**) and *Gata2^{cko}* (**J**) embryos. Analysis of the serotonergic neuron markers *Lmx1b* (**C**,**D**, ISH), *Fev* (**G**,**H**, ISH) and 5-HT (**K**,**L**, IHC) on adjacent coronal sections of E13.5 WT and *Gata2^{cko}* mutants. In I-L, higher magnification views from the r1 area are presented. Scale bars: 100 μm.

Analysis of midbrain GABAergic neuron subpopulations in perinatal *Gata2^{cko}* mutants demonstrates differential requirements for Gata2

To study whether the cells with the transformed neurotransmitter identity contribute to the maturing brain, we analyzed *Gata2^{cko}* mutants shortly before birth. At E17.5 and E18.5, the gross brain morphology of the *Gata2^{cko}* mutants was similar to that of the wild type. In sagittal sections, we observed loss of expression of the GABAergic markers *Gad1*, *Gata3* and *Pitx2* in the *Gata2^{cko}* midbrain, in both the dorsal and ventral brain regions (see Fig. S5 in the supplementary material). By contrast, abundant expression of GABAergic marker genes was detected in r1. Thus, consistent with the loss of GABAergic precursor cells in the midbrain at earlier stages of development, inactivation of *Gata2* leads into a specific loss of GABAergic neurons in the perinatal midbrain but not in r1.

To examine the development of the GABAergic neurons in the ventral midbrain, we performed ISH with *Gad1* and *Slc17a6* probes and IHC with anti-tyrosine hydroxylase (TH) antibodies on adjacent coronal sections of E18.5 embryos. Loss of *Gad1* and *Gata3* with concomitant upregulation of *Slc17a6* expression was observed in most regions of the midbrain (Fig. 7A-H; data not shown). Thus, the cells with the transformed neurotransmitter phenotype were still able to survive in the maturing brain. Strikingly, *Gad1* and *Gata3* expression in the ventrally located VTA and SNpr was unaltered. No increase in *Slc17a6* expression was observed in these nuclei either. Therefore, unlike in the rest of the midbrain, GABAergic neurons associated with the ventral DA nuclei appear normal in the *Gata2^{cko}* mutants.

To test the hypothesis that the GABAergic neurons of VTA and SNpr were derived from the midbrain neuroepithelium late in development, we analyzed *Gad1* and *Gata3* expression in the

ventral midbrain at E13.5 and E15.5, after the neurogenic period of this brain region (Altman and Bayer, 1981). Few *Gad1*- or *Gata3*-expressing cells were detected at E15.5 in the VTA-SNpr area of wild-type or *Gata2*^{cko} embryos (Fig. 7I-L,M-P; see Fig. S3 in the supplementary material). Thus, GABAergic neurons associated with the DA nuclei appear late in development.

DISCUSSION

Despite their importance in multiple aspects of behavior (Behbehani, 1995; Fields et al., 2007; Brandao et al., 2008), development of the midbrain GABAergic neurons is poorly understood. Here, we mapped the regions of the embryonic midbrain that give rise to GABAergic neurons. We identified *Gata2* as the first post-mitotic selector gene required for GABAergic identity in the midbrain. Finally, our results demonstrate differences in the regulatory pathways and developmental history of the GABAergic neurons in the midbrain-r1 region.

Gata2 marks regions of midbrain GABAergic neurogenesis

A useful dorsoventral fate map of the developing mouse midbrain was recently presented by Nakatani et al. (Nakatani et al., 2007). We detected *Gata2* and *Gata3* expression in all midbrain domains where GABAergic neurons are produced. Our studies suggest a further refinement to the map of Nakatani et al. We propose that the Nkx2-2-positive m4 domain gives rise to both GABAergic and glutamatergic neurons, the latter being negative for Gata2 and positive for Pax6 expression (Fig. 8A). In the future, it will be important to further define the mature brain structures to which these neural progenitor and precursor subpopulations contribute.

In the ventricular zone, the pattern of colocalization of Ascl1, Helt and Gata2 suggests that during their maturation, the Ascl1^{+} Helt^{+} progenitors turn into Ascl1^{+} Gata2^{+} precursors. Consistent with this, inactivation of *Helt* resulted in a dramatic reduction in *Gata2* expression. Our results thus suggest that *Gata2* expression is activated in a *Helt*-dependent fashion as the neural progenitor cells turn into post-mitotic GABAergic neuron precursors and leave the ventricular zone. The ventral m5 domain appears to be an exception, as here *Helt* was not essential for *Gata2* expression (Fig. 8B). Regulation of *Gata2* expression by an as yet unidentified mechanism in m5 is likely to explain the more severe phenotype of *Gata2*^{cko}, as compared with *Helt*, mutants.

Gata2 is a selector gene for GABAergic neuron identity of the post-mitotic midbrain precursors

Gata2 has been proposed to inhibit neural progenitor cell proliferation and promote post-mitotic differentiation, a model consistent with its pattern of expression (El Wakil et al., 2006). However, our results suggest that there are no marked changes in embryonic brain morphology and layering, progenitor proliferation, or neurogenic cell cycle exit in conditional *Gata2* mutants. Thus,

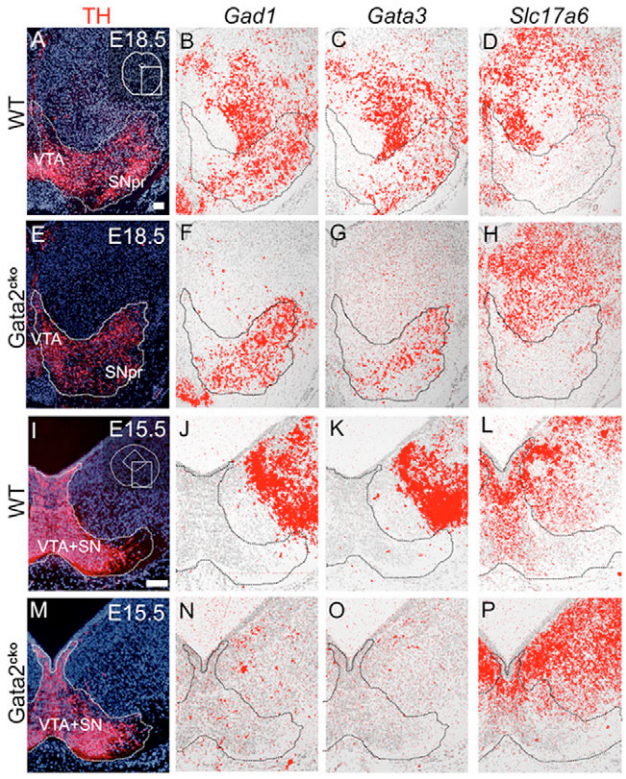

Fig. 7. Analysis of midbrain GABAergic neurons in *Gata2*^{cko} mouse mutants at late embryonic stages. (**A-H**) IHC analysis of tyrosine hydroxylase (TH) and ISH with *Gad1*, *Gata3* and *Slc17a6* probes on adjacent coronal sections of E18.5 wild-type (WT) and *Gata2*^{cko} midbrains. (**I-P**) TH, *Gad1*, *Gata3* and *Slc17a6* expression on adjacent coronal sections of E15.5 WT and *Gata2*^{cko} midbrains. SN, substantia nigra; SNpr, substantia nigra pars reticulata; VTA, ventral tegmental area. The dashed line delineates the TH-positive SNpr-VTA area. Scale bars: 100 μm.

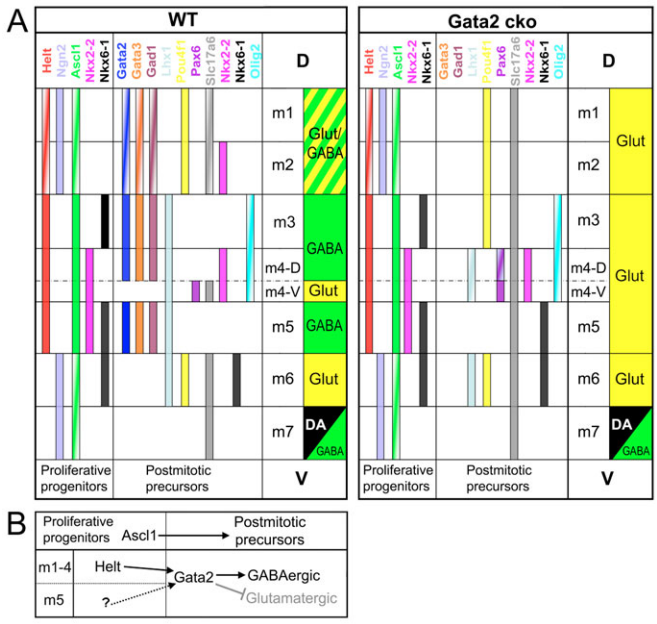

Fig. 8. Model for patterning and neurotransmitter identity determination in the midbrain. (**A**) Patterns of gene expression in the ventricular zone proliferative progenitors and post-mitotic precursors in the midbrain of wild-type (WT) and *Gata2*^{cko} mouse embryos. The m4 domain is divided into dorsal (D) and ventral (V) parts. (**B**) Model of the transcription factor functions regulating GABAergic neurogenesis. Cell cycle exit of GABAergic progenitors is primarily regulated by *Ascl1*, whereas the neurotransmitter identity is independently controlled by sequential functions of *Helt* and *Gata2*. In m5, *Helt* is not essential for *Gata2* expression, which may be activated by additional mechanisms.

although *Gata2* may still contribute to the cell cycle exit of neuronal progenitors, it is dispensable in this respect at least in the midbrain.

By contrast, the *Gata2* mutant neural precursors in the midbrain appear to undergo a complete cell fate transformation. Several genes, the expression of which is normally activated in the post-mitotic GABAergic precursors, failed to be expressed in the *Gata2* mutants. In addition, *Nkx6-1*, the expression of which is normally downregulated upon GABAergic differentiation in the m5 domain, continued to be expressed in the conditional *Gata2* mutants. Instead of the GABAergic phenotype, the *Gata2*-deficient midbrain precursor cells activate expression of glutamatergic marker genes. In the *Gata2* mutants, *Slc17a6* expression was observed throughout the midbrain, whereas *Pou4f1* expression was not ectopically activated in m4. By contrast, expression of Pax6 expanded in the m4 domain of *Gata2* mutants. As we showed that Pax6 is specifically expressed in glutamatergic precursors in m4, these results together suggest that in the conditional *Gata2* mutants, the identity of m4 is retained but the GABAergic m4 precursors switch to a glutamatergic fate. Also, in other parts of the midbrain, the *Gata2*-deficient precursors still have regional characteristics despite the transformation of their neurotransmitter identity.

Importantly, all the observed changes in gene expression take place in the post-mitotic precursors and neurons in the intermediate and marginal zones, whereas the proliferative progenitor cells and proneural gene expression in the ventricular zone are unaffected. This is in a clear contrast to the function of *Helt* in the ventricular zone, which has been shown to support GABAergic neurogenesis partly by repressing *Ngn2* expression in the progenitors and thereby glutamatergic neuron production (Nakatani et al., 2007). In addition to repressing *Ngn2*, *Helt* activates GABAergic gene expression (Miyoshi et al., 2004; Guimera et al., 2006; Nakatani et al., 2007), and *Gata2* appears to be important for this function. Thus, the expression and function of *Gata2* in the midbrain are analogous to those of the bHLH transcription factor *Ptf1a*, which acts as a post-mitotic selector gene of the GABAergic identity in the developing cerebellum and spinal cord (Glasgow et al., 2005; Hoshino et al., 2005; Mizuguchi et al., 2006).

Developmental diversity of the midbrain GABAergic neurons

In the perinatal *Gata2* mutants, all the midbrain GABAergic neuron subpopulations were transformed to a glutamatergic phenotype, except for the GABAergic neurons associated with the DA neurons in the VTA and SNpr. This was highly unexpected because no GABAergic precursor cells were found at any dorsoventral level of the midbrain at the earlier stages of development. There are at least three possible explanations for this. Firstly, there could be incomplete recombination of the $Gata2^{flox}$ allele by $En1^{Cre}$. We find this unlikely because we could not see any *Gata2* transcripts or protein in the $Gata2^{cko}$ mutants analyzed at several stages. Also, no evidence for mosaic inactivation of *Gata2* was observed in VTA, SNpr, or other regions of the midbrain. Secondly, the remaining GABAergic neurons could be born in a region of the midbrain that does not require *Gata2* and was missed in our analysis. We cannot completely rule out this possibility, but nor is there any evidence for it, as no *Gad1*- or *Gata3*-positive cells were observed at any anteroposterior or dorsoventral level of the mutant midbrain at E11.5-15.5. Finally, the GABAergic neurons of the VTA and SNpr could be derived from neuroectoderm outside the midbrain. The late appearance of the VTA and SNpr GABAergic neurons (~E15.5) and their strikingly normal development in the $Gata2^{cko}$ mutants are consistent with this hypothesis. Possible sources of the VTA and SNpr GABAergic neurons include r1 and the diencephalon. However, cell lineage-tracing experiments are needed to unambiguously determine the origin of these ventral-most GABAergic neurons.

Gata2 is dispensable for GABAergic, but essential for serotonergic, neuron development in rhombomere 1

Similar to in the midbrain, *Gata2* and *Gata3* are expressed in the GABAergic precursors of r1. Strikingly, however, we observed abundant GABAergic neurons in r1 of the *Gata2* conditional mutants, despite the complete loss of *Gata2* in this brain region. Interestingly, the hindbrain GABAergic precursors continued to express *Gata3*. This suggests that *Gata3* expression is, perhaps, not directly regulated by *Gata2* in the GABAergic precursors in r1. Analyses of *Gata3* mutants and, possibly, *Gata2*; *Gata3* double mutants, are needed to determine whether *Gata3* can possibly compensate for the loss of *Gata2* specifically in the r1 GABAergic precursor cells.

The development of rostral serotonergic neurons has also been shown to be regulated by *Gata2*. Craven et al. used explant cultures of *Gata2*-null mutant neuroepithelium to demonstrate a requirement for *Gata2* for the differentiation of serotonin-positive cells in r1 (Craven et al., 2004). Consistent with this, our results show loss of serotonergic neurons in the conditional *Gata2* mutants. In contrast to the *Gata2* mutant explant cultures, our results suggest that loss of *Gata2* also leads to downregulation of *Gata3* in the serotonergic neuron precursors. This discrepancy might be explained by distorted tissue architecture of the cultured explants and by the continued expression of *Gata3* in the nearby GABAergic precursors in r1. It will be of interest to determine whether *Gata2* also acts as a post-mitotic selector gene in the serotonergic neuron lineage, similar to its role in the developing midbrain GABAergic neuron precursors.

Conclusion

GABAergic versus glutamatergic neuron identity appears to be regulated by different genetic cascades in different regions of the CNS. Here we show specific requirement for the transcription factor Gata2 as a cell-fate selector, and locate it in the gene hierarchy regulating development of the GABAergic neurons in the midbrain (Fig. 8B). Our study also further elucidates how regional identities are generated in the distinct midbrain GABAergic neuron subpopulations. Understanding the development, molecular identity and functional characteristics of these diverse neurons might lead to better diagnostics and treatment of several forms of neurological and psychiatric disease.

We thank Eija Koivunen and Raija Savolainen for technical assistance; Francois Guillemot for the *Ascl1* mutant mice; Ryochiro Kageyama for the anti-Helt antibody; Jeannette Nardelli for the *Gata2* expression vector; Siew-Lan Ang, Qiufu Ma, Horst Simon, Vassilis Pachnis and Peter Gruss for cDNA probes; and Mart Saarma, Tapio Heino and Claudio Rivera for comments. This study was supported by funding from the Marie Curie ESTP (M.H.), Academy of Finland, Biocentrum Helsinki and Sigrid Juselius Foundation.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/2/253/DC1

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